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ARTICLE

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Inflammatory gene expression in monocytes of patients with schizophrenia: overlap and difference with bipolar disorder. A study in naturalistically treated patients

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Abstract

Accumulating evidence indicates an activated inflammatory response system as a vulnerability factor for schizophrenia (SZ) and bipolar disorder (BD). We aimed to detect a specific inflammatory monocyte gene expression signature in SZ and compare such signature with our recently described inflammatory monocyte gene signature in BD. A quantitative-polymerase chain reaction (Q-PCR) case-control gene expression study was performed on monocytes of 27 SZ patients and compared to outcomes collected in 56 BD patients (all patients naturalistically treated). For Q-PCR we used nine 'SZ specific genes' (found in whole genome analysis), the 19 BD signature genes (previously found by us) and six recently described autoimmune diabetes inflammatory monocyte genes. Monocytes of SZ patients had (similar to those of BD patients) a high inflammatory set point composed of three subsets of strongly correlating genes characterized by different sets of transcription/MAPK regulating factors. Subset 1A, characterized by *ATF3* and *DUSP2*, and subset 1B, characterized by *EGR3* and *MXD1*, were shared between BD and SZ patients (up-regulated in 67% and 51%, and 34% and 41%, respectively). Subset 2, characterized by *PTPN7* and *NAB2* was *up-regulated* in the monocytes of 62% BD, but *down-regulated* in the monocytes of 48% of SZ patients. Our approach shows that monocytes of SZ and BD patients overlap, but also differ in inflammatory gene expression. Our approach opens new avenues for nosological classifications of psychoses based on the inflammatory state of patients, enabling selection of those patients who might benefit from an anti-inflammatory treatment.

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Introduction

We (Padmos *et al*. 2008*a*) recently described a sensitive quantitative polymerase chain reaction (Q-PCR) assay system to detect the pro-inflammatory state of circulating monocytes of naturastically treated patients with bipolar disorder (BD) patients and detected in the monocytes a coherent, mutually correlating set of 19 aberrantly expressed inflammatory genes ('a gene signature or fingerprint'), supporting the concept of an activated inflammatory response system (IRS) in mood disorders (Smith & Maes, 1995).

Since the concept of an activated IRS also extends to schizophrenia (SZ) (Smith & Maes, 1995), we hypothesized that the same or a similar abnormal inflammatory gene fingerprint could also be found in monocytes of patients with SZ and we decided to test

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for the 19 aberrantly expressed 'bipolar signature genes' in the circulating monocytes of naturalistically treated SZ patients. In addition, we searched for new 'schizophrenia inflammatory genes' using Affymetrix whole genome expression profiling (Affymetrix, USA) on monocytes of SZ patients and selected those genes which were markedly aberrantly expressed and clearly involved in inflammation (yielding 15 'new' genes, for details see Results section).

Autoimmune diabetes, thyroiditis and gastritis are about three times more prevalent in BD (Padmos *et al*. 2004), whereas autoimmune thyrotoxicosis and Sjogren's disease are more prevalent in SZ (Eaton *et al*. 2006). Given our recently reported overlap in monocyte gene expression signatures between BD and autoimmune diabetes, we additionally included in our analysis six 'specific autoimmune diabetes signature monocyte genes (Padmos *et al*. 2008*b*)' to be complete.

Thus, using Q-PCR, we validated for this report the abnormal expression of 34 monocyte activation genes in 27 patients with SZ [compared to monocytes of 32 age-/gender-matched healthy controls (HC)] and 56 patients with BD [42 patients of the previously reported series (Padmos *et al*. 2008*a*) *plus* 14 new cases, altogether compared to monocytes of 48 age-/gender-matched HC].

Patients and methods

Patients with SZ

Patients with SZ were diagnosed according to the DSM-IV criteria and recruited at the Department of Psychiatry of the Erasmus Medical Center in Rotterdam. All patients were in-patients. Patients were diagnosed with SZ according to the DSM-IV criteria after a Comprehensive Assesment of Symptoms and History (CASH) interview (Andreasen *et al*. 1992) and by consensus between two senior psychiatrists who were blinded to the results. For patients with symptoms for <6 months, a final diagnosis was made after 6 months to comply with the DSM-IV criterion. All patients were acutely psychotic.

The SZ patients were almost all recent onset cases and had a median duration of illness of only 0.3 yr (range 0–3 yr). No cases suffered from any other severe medical illness (including infections and allergies), verified with a medical history assessment and routine laboratory testing (Hb, Ht, leukocyte count, blood smear and kindney/liver function) on admission.

For Affymetrix microarray analysis (searching for aberrantly expressed genes in monocytes) two monocyte pools of SZ patients were used. Each pool was compared to a monocyte pool of age- and gender-matched HC (pools were used for minimizing inter-individual differences in mRNA expression and to reduce costs for this expensive methodology). Patient pool 1 consisted of four male cases, aged 22, 26, 27 and 20 yr, patient pool 2 consisted of three male cases, aged 17, 19 and 27 yr. Pools 1 and 2 were compared to two HC pools of 2×2 males, aged between 22 and 26 yr.

For Q-PCR (verifying the found genes) 27 additional SZ patients diagnosed according to DSM-IV criteria were recruited at the Department of Psychiatry of the Erasmus Medical Center in Rotterdam.

All but one of the patients received antipsychotic medication at the time of blood draw; none of the patients were drug naive. The demographics, duration of illness and drug usage of the patients used in Q-PCR are summarized in Table 1.

For the Q-PCR on SZ patients we used a control group of 32 HC, who were age-/gender-matched to the SZ patients. These were recruited from enrolling laboratory staff, medical staff and medical students (Table 1). The inclusion criteria for HC were an absence of any psychiatric and autoimmune disorder and an absent history of these disorders in first-degree family members. HC had to be in self-professed good health and free of any obvious medical illness for at least 2 wk prior to blood draw, including acute infections and allergic reactions.

Patients with BD

In total, 56 outpatients with DSM-IV bipolar I or II disorder were recruited from two studies, i.e. the Dutch site of the former Stanley Foundation Bipolar Network (SFBN), an international multi-centre research programme described in detail previously (Suppes *et al*. 2001) ($n=19$ patients) and from an ongoing Dutch twin study on BD described in detail by Vonk *et al*. (2007) ($n=37$). Characteristics of BD patients are given in Table 1. Diagnosis was also made by means of the SCID. Present mood state was evaluated via the Young Mania Rating Scale (YMRS) and the Inventory for Depressive Symptomatology (IDS). The BD patients did not have another severe medical illness, verified by medical history assessment.

Since age and gender differed between our BD and SZ patients (Table 1) we compared outcomes of the BD group to those of an extra group of 48 HC, who were age-/gender-matched to the BD patients (Table 1). For inclusion criteria for HC see earlier.

The Medical Ethical Review Committee of the University Medical Center Utrecht (BD patients) and the Medical Ethical Review Committee of the Erasmus

Table 1. Characteristics of schizophrenia and bipolar patients and their respective healthy controls used for Q-PCR

	Schizophrenia	Matched healthy controls
Group size	27	32
Age (yr)[a]	27 (17–59)	27 (21–47)
Gender		
Male	22 (81%)	25 (78%)
Female	5 (19%)	7 (22%)
Duration illness (yr)	0.3 (0–3)	
Age of onset	26 (17–58)	
Medication		
Typical antipsychotics	10 (37%)	
Atypical antipsychotics	15 (56%)	
Other	1 (4%)	
None	1 (4%)	
	Bipolar disorder[bc]	**Matched healthy controls**
Group size	56	48
Dutch twin study	37	
Dutch site SFBN	19	
Age (yr)[a]	42 (26–61)	42 (23–57)
Gender		
Male	22 (39%)	20 (42%)
Female	34 (61%)	28 (58%)
Duration illness (yr)	16 (3.5–40)	
Age of onset (yr)	26 (6–49)	
Medication		
Lithium	32	
Antipsychotics	8	
Antipsychotics and lithium	5	
Other	11	

SFBN, Stanley Foundation Bipolar Network.

[a] Mean (range).

[b] The bipolar patients did not have a history of drug or alcohol dependency for at least 6 months; this was not known for the schizophrenia patients.

[c] Data on 42 of these 56 patients have been published previously (Padmos *et al*. 2008*a*).

MC Rotterdam (SZ patients) approved the studies. Written informed consent was obtained from all participants after a complete description of the study had been given.

Laboratory methods

Blood collection and preparation

Blood (drawn in the morning) was collected in clotting tubes for serum preparation (stored at −80 °C) and in sodium-heparin tubes for immune cell preparation. From the heparinized blood, peripheral blood mononuclear cell (PBMC) suspensions were prepared in the afternoon by low-density gradient centrifugation, as previously described in detail (Knijff *et al*. 2006), within 8 h to avoid activation of the monocytes (erythrophagy). PBMCs were frozen in 10% dimethylsulfoxide and stored in liquid nitrogen. This enabled us to test patient and control immune cells in the same series of experiments later.

Isolation of monocytes

CD14-positive monocytes were isolated from frozen PBMCs by a magnetic cell sorting system (Miltenyi Biotec, Germany). The purity of monocytes was >95% (determined by morphological screening after Trypan Blue staining and fluorescent-activated cell sorting). As previously reported, positive *vs.* negative selection of immune cells did not influence gene expression profiles (Lyons *et al*. 2007).

Affymetrix whole genome gene expression profiling

RNA was isolated from purified monocytes using RNAeasy columns according to the manufacturer's instructions (Qiagen, USA) and as previously described (Staal *et al*. 2004). Fragmented cRNA was hybridized to U95Av2 microarrays according to the manufacturer's instructions (Affymetrix). For all experiments, the 5′/3′ ratios of GAPDH were ⩽2 (usually 0.9–1.1).

Q-PCR

RNA was isolated from monocytes as described earlier. To obtain cDNA for Q-PCR, 1 μg RNA was reversed-transcribed using the cDNA high-capacity cDNA Reverse Transcription kit (Applied Biosystems, USA). Q-PCR was performed as previously described in detail by Staal *et al*. (2004) and in the legend of Table 2.

Statistics

Scanned microarray images were analysed using Affymetrix Microarray Suite 4.2 software. Further analysis was performed using RMA software, modification by de Ridder (de Ridder *et al*. 2006) and Ingenuity Systems (www.ingenuity.com) software. Statistical analysis was performed using the SPSS 15.0 package for Windows (SPSS Inc., USA). Data were tested for normal distribution using the Kolmogorov–Smirnov test. Depending on the distribution pattern and the total number of subjects, parametric (normal distribution and ⩾50 subjects) or non-parametric tests

Table 2. Q-PCR analysis of monocytes of bipolar (BD) patients [$n=56$, 42 from a previous study (Padmos *et al*. 2008*a*) plus 14 new cases) and schizophrenia (SZ) patients ($n=27$) compared to healthy control (HC) values (HC SZ: $n=32$; HC BD: $n=48$), set at 1-fold.

	Schizophrenia		Bipolar disorder	
Genes selected by whole genome screening in this study in schizophrenia				
EGR3	5.36[a]	<0.01[c]	2.52	0.16
MXD1	1.49	<0.01	1.43	0.06
MAFF	5.10	<0.01	2.95	0.01
F3	5.56	<0.01	1.87	0.02
SERPINB2	1.84	<0.01	1.10	0.06
THBS	4.31	<0.01	2.02	0.05
EREG	7.36	<0.01	2.31	<0.01
CXCL3	3.99	<0.01	3.33	<0.01
RGC32	0.85[b]	0.07	2.61	<0.01
Genes selected in a previous study on bipolar patients (Padmos *et al*. 2008*a*)				
DUSP2	5.36	<0.01	4.96	<0.01
ATF3	3.50	<0.01	3.55	<0.01
MAPK6	1.21	<0.01	1.80	<0.01
PDE4B	3.91	<0.01	3.00	<0.01
IL6	7.89	<0.01	5.39	<0.01
IL1B	9.20	<0.01	6.45	<0.01
TNF	3.91	<0.01	1.87	<0.01
TNFAIP3	3.22	<0.01	2.31	<0.01
BCL2A1	2.39	<0.01	3.30	<0.01
PTX3	2.51	<0.01	2.63	<0.01
PTGS2	4.34	<0.01	3.20	<0.01
CCL7	1.12	<0.01	8.47	<0.01
CDC42	1.49	<0.01	1.99	<0.01
CCL20	23.53	<0.01	10.63	<0.01
CXCL2	3.76	<0.01	5.31	<0.01
CCL2	1.60	<0.01	3.83	<0.01
CCR2	0.85	0.53	0.62	0.10
NAB2	0.76	0.21	2.58	<0.01
EMP1	0.97	0.88	2.19	<0.01
Genes selected in previous study on autoimmune diabetes (Padmos *et al*. 2008*b*)				
PTPN7	0.78	0.04	2.04	<0.01
CD9	1.49	0.59	2.16	<0.01
STX-1A	0.64	0.22	3.04	<0.01
DHRS3	1.08	0.51	1.87	0.08
FABP5	1.08	0.75	1.21	0.09
HSPA1A	1.06	0.93	0.79	0.40

Q-PCR was performed with Taqman Universal PCR mastermix (Applied Biosystems, USA). All Taqman probes and consensus primers were pre-formulated and designed by Applied Biosystems (Assays on Demand, see Supplementary Table 1, online). PCR conditions were 2 min at 50 °C, 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C, and finally 1 min at 60 °C. PCR amplification of the reference gene *ABL* was performed for each sample to allow normalization between the samples. *ABL* was chosen as a reference gene because it was previously shown that *ABL* was the most consistently expressed reference gene in haematopoietic cells (Beillard *et al.* 2003). The quantitative value obtained from Q-PCR is a cycle threshold (C_t). The fold change values between different groups were determined from normalized C_t values (C_t gene – C_t housekeeping gene), by the $\Delta\Delta C_t$ method

(skewed distribution or <50 subjects) were used. Bonferroni correction for multiple testing was used for the Affymetrix data (since this was a non-hypothesis-driven approach). Correction for multiple testing was not used for the analysis of the Q-PCR data, because we focused on the effect of specific genes found in the Affymetrix analysis. The specific tests used are mentioned in the table notes and figure legends.

Results

Whole genome expression profiling of potential inflammatory biomarker genes in monocytes of SZ patients

Affymetrix microarray analysis was performed to search for aberrantly expressed genes in monocytes on two monocyte pools of naturalistically treated SZ patients. Each pool was compared to a monocyte pool of age- and gender-matched HC (pools were used for minimizing inter-individual differences in mRNA expression and to reduce costs for this expensive methodology). All raw data obtained by Affymetrix analysis are available as MIAMExpress submission (http://www.ebi.ac.uk/miamexpress/).

We analysed the data using a modified RMA analysis (de Ridder *et al*. 2006) and considered for Ingenuity analysis genes which were >2-fold statistically differentially expressed ($p<0.01$, corrected for multiple testing) between SZ patients and HC. This resulted in 298 discriminating genes (185 up-regulated and 113 down-regulated). Major pathways found in Ingenuity analysis were pathways involved in inflammatory and immune mediated disease. To select for genes which could serve as potential biomarkers for the 'schizophrenia inflammatory condition', we took the top genes from the up and down list, which were statistically >3.5-fold significantly differentially expressed between SZ and HC with the purpose of only identifying strongly discriminating genes. This resulted in 22 overexpressed genes. None of the genes was >3.5-fold lower expressed [the first gene of the list of the lower-expressed genes was *CCR2*, which was 2.9-fold lower expressed, but this gene had already been selected in our previous bipolar study (Padmos *et al*. 2008*a*). Because we were searching for regulators and biomarkers of inflammation, out of these 22 aberrantly expressed genes we selected only genes clearly involved in inflammation. This resulted in 14 selected aberrantly expressed genes for SZ, and it is of note that five of these genes had previously been found overexpressed in BD patients, i.e. *PDE4B*, *IL1*, *PTGS2/COX2*, *CCL20* and *CXCL2* (Padmos *et al*. 2008*a*), pointing to a strong overlap of inflammatory set points between monocytes of BD and SZ. In sum, nine new 'schizophrenia specific' up-regulated genes (*MXD1*, *F3*, *MAFF*, *EGR3*, *THBS*, *SERPINB2/PAI-2*, *RGC32*, *EREG*, *CXCL3*) were finally selected and we included these nine new genes together with the 19 aberrantly expressed 'bipolar signature genes' and the six 'autoimmune diabetes signature genes' in the validating Q-PCR analysis of the 27 SZ patients, 56 BD patients [42 patients of the previously reported study of Padmos *et al*. (2008*a*) plus 14 new cases] and their 32 and 48 matched HC respectively.

Q-PCR analysis of monocytes of SZ and BD patients

Table 2 (and Supplementary Table 1, available online) show that of the 34 genes tested, the mRNA expression levels of 25 genes were significantly different ($p<0.05$ by ANCOVA, corrected for age and gender) in the monocytes of the 27 SZ patients compared to HC, while in the monocytes of the 56 BD patients 27 genes were significantly differently expressed. Data obtained in Q-PCR on the mRNA expression levels of the various genes in the patient groups correlated very strongly to those obtained in the above-described Affymetrix analysis (SZ: $r=0.708$; BD: $r=0.663$, Spearman's ρ).

(*Table 2 footnote continued*)

($2^{-\Delta\Delta C_t}$, User Bulletin 2, Applied Biosystems, USA). To correct for inter-assay variance, we set the mean of the studied genes found in the healthy control groups in the same assay for each gene to 1 ($\Sigma\Delta C_t$: HC=0, $2^{-0}=1$). The fold change values of the genes in patients' monocytes were expressed relative to this set mean of 1.

Data are expressed relative to this HC value. HC SCZ: $n=32$; HC BD: $n=48$.

[a] Values >1: patients have a higher expression than control group.

[b] Values <1: patients have a lower expression than control group.

[c] *P* tested by univariate ANCOVA *vs*. control subjects; age and gender are included in this model.

Purity of monocytes was >90% (as determined by morphology on each sample) and >92% as determined by fluorescent assisted cell sorting analysis. Yield of monocytes was 28% (±10%) in the patient groups group and 21% (±8%) for the HC group (n.s.) of the Ficoll-isolated peripheral blood mononuclear cells.

The ΔC_t values are available in Supplementary Table 1 (online).

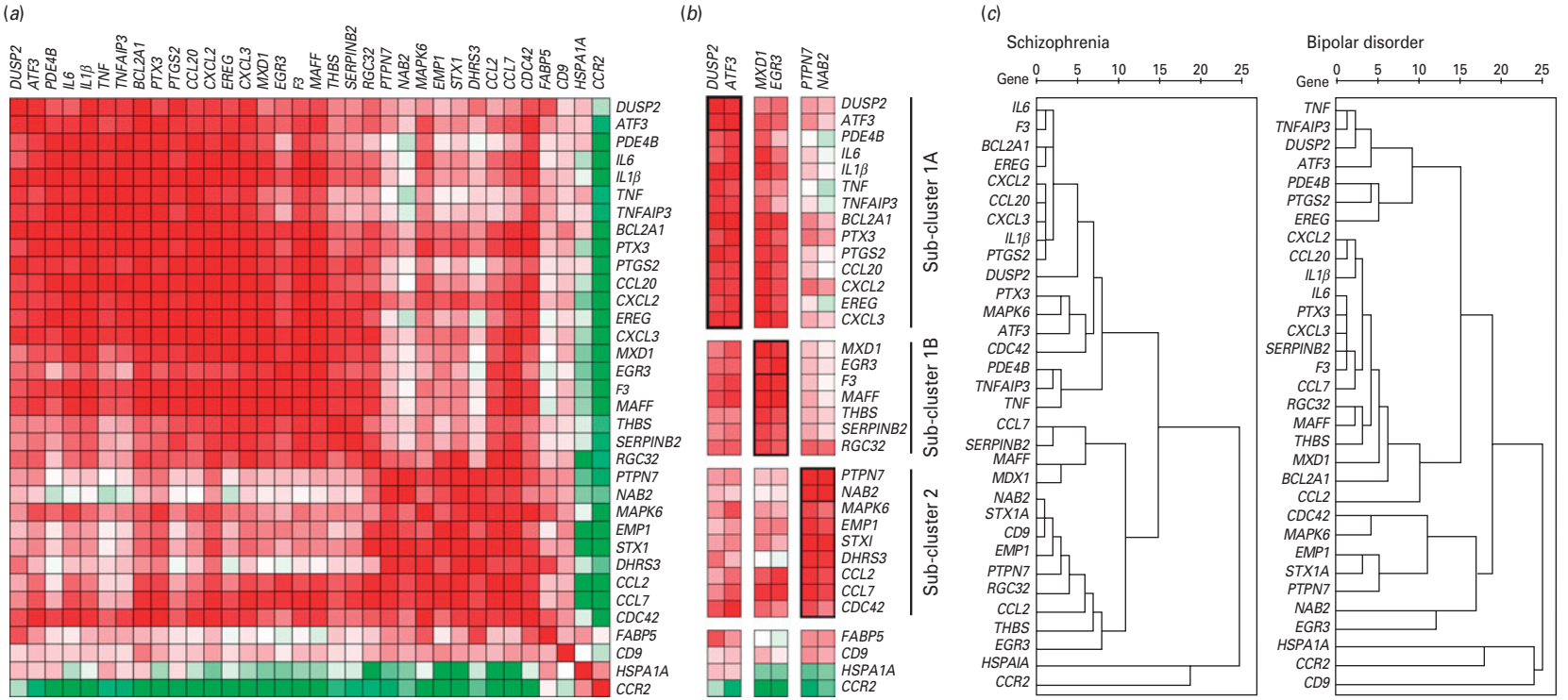

Fig. 1. Heat map of gene correlation. Correlation of expression of the various genes; data represent Spearman's correlation coefficients, tested on the relative mRNA expression of the genes in 83 individuals: 56 bipolar patients, 27 schizophrenia patients. Significant positive correlations ($p < 0.05$) are given by the red scale (darkest red are correlations > 0.60), significant negative correlations are given by the green scale. White fields are not significant. (*a*) The correlations of all tested genes to each other are shown. (*b*) Three sets of MAPK regulators/transcription factors have been extracted from panel (*a*), namely *DUSP2/ATF3*, *MXD1/EGR3* and *PTPN7/NAB2* and correlations to the other genes are shown. Note that (1) *DUSP2/ATF3* correlate strongest to sub-cluster 1A genes (and weaker to the other subsets of genes), (2) *MXD1/EGR3* correlates strongest to sub-cluster 1B genes and many of the sub-cluster 1A genes (but weaker to *DUSP2/ATF3*) and (3) that *PTPN7/NAB2* correlates strongest to sub-cluster 2 genes. (*c*) Dendrograms of schizophrenia and bipolar disorder.

Table 3. Fold change values of all genes grouped by cluster

	Schizophrenia		Bipolar disorder	
Cluster 1A				
DUSP2	**5.36**	**<0.01**	**4.96**	**<0.01**
ATF3	**3.50**	**<0.01**	**3.55**	**<0.01**
PDE4B	3.91	<0.01	3.00	<0.01
IL6	7.89	<0.01	5.39	<0.01
1L1B	9.20	<0.01	6.45	<0.01
TNF	3.91	<0.01	1.87	<0.01
TNFAIP3	3.22	<0.01	2.31	<0.01
BCL2A1	2.39	<0.01	3.30	<0.01
PTX3	2.51	<0.01	2.63	<0.01
PTGS2	4.34	<0.01	3.20	<0.01
CCL20	23.53	<0.01	10.63	<0.01
CXCL2	3.76	<0.01	5.31	<0.01
EREG	7.36	<0.01	2.31	<0.01
CXCL3	3.99	<0.01	3.33	<0.01
Cluster 1B				
MXD1	**1.49**	**<0.01**	1.43	0.06
F3	**5.56**	**<0.01**	**1.87**	**0.02**
MAFF	**5.10**	**<0.01**	**2.95**	**0.01**
EGR 3	**5.36**	**<0.01**	2.52	0.16
THBS	4.31	<0.01	2.02	0.05
PAI-2	1.84	<0.01	1.10	0.06
RGC32	0.85	0.07	2.61	<0.01
Cluster 2				
hePTP	**0.78**	**0.04**	**2.04**	**<0.01**
NAB2	**0.76**	**0.21**	**2.58**	**<0.01**
MAPK6	**1.21**	**<0.01**	**1.80**	**<0.01**
EMP1	0.97	0.88	2.19	<0.01
STX-1A	0.64	0.22	3.04	<0.01
DHRS3	1.08	0.51	1.87	0.08
CCL2	1.60	<0.01	3.83	<0.01
CCL7	1.12	<0.01	8.47	<0.01
CDC42	1.49	<0.01	1.99	<0.01
FABP5	1.08	0.75	1.21	0.09
CD9	1.49	0.59	2.16	<0.01
HSPA1A	1.06	0.93	0.79	0.40
CCR2	0.85	0.53	0.62	0.10

The quantitative value obtained from Q-PCR is a cycle threshold (C_t). The fold change values between different groups were determined from the normalized C_t values (C_t gene – C_t housekeeping gene), via the $\Delta\Delta$ C_t method (User Bulletin, Applied Biosystems). The fold change of HC was set to 1.

Data are expressed relative to this HC value. HC SCZ: $n=32$; HC BD: $n=48$.

Values >1: patients have a higher expression than control group. Boxes indicate significantly up-regulated.

Values <1: patients have a lower expression than control group. Grey shaded box indicates significantly down-regulated.

p tested by univariate ANCOVA *vs.* control subjects; age and gender are included in this model.

There were no differences in mRNA gene expression for the different groups of HC (Supplementary Table 1).

The aberrantly expressed genes were mostly shared between the two disorders but also in part not shared. Of the nine 'schizophrenia specific' genes, eight were confirmed by Q-PCR in SZ (*RGC32* appeared not to be higher expressed); while in BD patients five of these genes were significantly higher expressed (*MAFF*, *F3*, *EREG*, *CXCL3*, *RGC32*).

Of the 19 'bipolar signature genes' 16 were statistically significantly up-regulated in SZ, while for three genes statistical significance was not reached (*CCR2*, *NAB2*, *EMP1*). Confirming our previous data, we found almost all 19 'bipolar signature genes' (apart from *CCR2*) statistically significantly overexpressed in this extended set of BD patients.

Of the six 'autoimmune diabetes signature genes', five were not aberrantly expressed in SZ, while *PTPN7* was statistically significantly down-regulated. Interestingly, in the BD sample 3/6 genes were up-regulated, including *PTPN7*.

Cluster analysis and identification of sub-clusters in the pro-inflammatory signature

To study their mutually inter-dependent state in expression, we subsequently performed a cluster analysis on the Q-PCR data. The heat map and dendrograms of this analysis are given in Fig. 1*a*. In sum, expression levels of virtually all genes correlated to each other, yet two sub-clusters of mutually very strongly correlating genes (correlation coefficient >0.60) could clearly be identified (two major red blocks in the figure), each predominantly correlating to a different set of transcription/MAPK regulating factors, i.e. *ATF3/DUSP2* and *PTPN7/NAB2*, respectively. These two sets of transcription factors were mutually, but not strongly, correlated (Fig. 1*b*).

The first sub-cluster correlating to *ATF3* and *DUSP2* consisted predominantly of various well-known inflammatory compounds, such as the pro-inflammatory cytokines *IL1*, *IL6* and *TNF*, the inflammatory compounds *PTGS2/COX2* and *PTX3*, various inflammatory chemokines (*CCL20*, *CXCL2*, *CXCL3*) and *PDE4B* (Fig. 1*b*).

The second sub-cluster (further indicated as sub-cluster 2) correlating to *PTPN7* and *NAB2* consisted predominantly of various adhesion/motility/chemotactic factors, such as *EMP1*, *CDC42*, *CCL2* and *CCL7* (Fig. 1*b*).

In careful analysis (Fig. 1*b*, Supplementary Fig. 1, online), sub-cluster 1 contained a further sub-cluster

Table 4. The prevalence of sub-clusters in bipolar patients, schizophrenia patients and healthy controls

	Cluster 1A	Cluster 1B	Cluster 2	Cluster 2
	Dusp2 and/or *ATF3* positivity	*MXD1* and/or *EGR3* positivity	*PTPN7* and/or *NAB2* positivity	*PTPN7* and/or *NAB2* negative
Schizophrenia	52% (14/27)[a]	41% (11/27)[a]	7% (2/27)[b]	48% (13/27)[ab]
Healthy controls	24% (7/29)	13% (4/30)	21% (6/29)	21% (6/29)
Bipolar disorder	67% (26/39)[a]	34% (12/36)	62% (24/39)[a]	5% (2/39)[a]
Healthy controls	24% (10/41)	22% (8/36)	32% (13/41)	25% (10/40)

[a] $p < 0.05$ *vs.* healthy controls.

[b] $p < 0.05$ *vs.* bipolar disorder.

Positive is defined as an mRNA expression >1 S.D. away of the mean level found in the healthy controls. Signature is defined on the transcription factors positive. p values are obtained from χ^2 test.

consisting of the transcription factors *EGR3* and *MXD1*, which were mutually strongly correlating to the transcription factors *MAFF* and *F3*, but more weakly to *ATF3/DUSP2* and *PTPN7/NAB2* (Fig. 1*b*, this sub-sub cluster is further indicated as sub-cluster 1B, the other remaining set being sub-cluster 1A).

The expression of the three sub-clusters in SZ and BD

Tables 3 and 4 show that sub-cluster 1A is expressed in the monocytes of both SZ and BD patients. If one defines sub-cluster 1A positivity as positive for *ATF3* and/or *DUSP2* (i.e. an expression level higher than the mean ± 1 S.D. of the HC values) 67% of BD and 52% of SZ patients are positive *vs.* 24% and 24% of their matched HC, respectively (Table 4).

With regard to sub-cluster 1B, Tables 3 and 4 show that SZ patients and BD patients are positive, but in lower proportions. Using the same type of definition as for sub-cluster 1A (but now for *EGR3* and/or *MXD1*), it appeared that SZ patients were significantly positive for 41% *vs.* 34% of BD patients (*vs.* 13% and 22% of their HC, Table 4).

With regard to sub-cluster 2, Tables 3 and 4 show that only BD patients show an up-regulation of these genes. Interestingly, SZ patients show a significant down-regulation of two transcription factors belonging to this sub-cluster, i.e. *PTPN7* and *NAB2* (significant for *PTPN7*), while MAPK6 is up-regulated (yet not to the same extent as in BD). It is noteworthy that the adhesion/motility factors *EMP1* and *STX1A* are down-regulated too. If one defines sub-cluster 2 positivity as positive for *PTPN7* and/or *NAB2* (see above for definition), even a significantly reduced expression in SZ *vs.* HC can be seen (7% *vs.* 21%), while in BD there is a significant increased expression (62% *vs.* 32%) (Table 4). Conversely, if sub-cluster 2 is defined as a 'negative' signature (Table 4, last column) a higher proportion of SZ patients is positive for such reduced expression (48%).

Relation of monocyte inflammatory gene expression to medication use, disease duration and disease activity

Medication use

To test for the influence of lithium and antipsychotics, we turned to the group of BD patients, since almost all patients with SZ were on antipsychotics; only one was not, but had used an antipsychotic in the past. Of the 56 BD patients, 32 were on lithium, eight were on antipsychotics, five used both and at the time of blood draw 11 were not on lithium or an antipsychotic but had used this medication in the past (>6 months ago). Table 5*a* shows the effects of the medications in this BD group. Use of lithium and antipsychotics either alone or in combination resulted in a significant decrease of *PDE4B*, but not of other genes (although there was a near significant trend for a decreasing effect of lithium and antipsychotics on other important signature 1A genes such as *IL1* and *TNF*).

With regard to sub-cluster 2 genes there was a near significant increasing trend of the use of antipsychotics in BD patients on genes such as *PTPN7*, *NAB2* and *STX1A* (data not shown). This increasing effect of antipsychotics on sub-cluster 2 genes was also reflected in the 'lifetime cumulative antipsychotic drug usage in haloperidol equivalents' which positively and significantly correlated to important sub-cluster 2 genes as *PTPN7* ($r = 0.55$), *HSPA1A* ($r = 0.61$) and *EMP1* ($r = 0.62$).

Table 5. Correlations of aberrant gene expression

(*a*) With medication use in bipolar patients

Medication	Genes	*p*		*B*	95% CI
Lithium	*PDE4B*	0.002	down	−12.302	−4.736 to −19.867
Antipsychotics	*PDE4B*	0.002	down	−16.613	−6.555 to −26.671
Both	*PDE4B*	0.005	down	−17.142	−5.468 to −28.817

Linear regression with lithium, antipsychotics and both medications were included in the model. The values of patients on the indicated drug are set to 1 in the model. *B*, Regression coefficient.

(*b*) With the mood status of bipolar patients

	Depressive ($n=9$) *vs.* Euthymic ($n=40$)			Manic ($n=7$) *vs.* Euthymic ($n=40$)		
Genes	*B*	95% CI	*p*	*B*	95% CI	*p*
CCL2	105.24	37.49–172.98	0.003	22.93	−44.81 to 90.68	0.500
STX1A	36.59	13.52–59.69	0.002	8.77	−14.30 to 31.83	0.449
DHRS	9.91	3.00–16.83	0.006	−0.28	−6.78 to 6.22	0.930
PTPN7	3.82	1.08–6.56	0.007	2.25	−0.49 to 4.99	0.106
MAPK6	2.76	0.83–4.69	0.006	2.29	0.36 to 4.22	0.021
EMP1	12.72	7.31–18.12	0.001	3.83	−1.58 to 9.24	0.161

Determination of the influence of mood on mRNA expression of molecules via ANCOVA analysis.
The values of patients with a euthymic mood are set to 1. *B*, Regression coefficient.

Duration of illness

The patients with SZ were in general recent onset cases and had a median duration of illness of only ∼2 months (Table 1). Effects of duration of illness were not noticeable.

The BD patients had a median duration of illness of 16 yr (range 3.5–40 yr, Table 1); in this latter group there was a weak, although significant correlation between disease duration and the expression of some of the signature genes: we found a weak positive correlation for *IL6*, *PTX3*, *CCL2* and *EMP1* ($r=0.30$–0.40) and a weak negative correlation for *HSPA1A* ($r=-0.30$), indicating a slightly stronger expression of part of the inflammatory fingerprint over years.

Disease quality and severity

In the SZ patients disease quality and severity (as expressed in the various PANSS scales) did not correlate to any of the various gene expression levels.

With regard to BD, we previously reported (Padmos *et al*. 2008*a*) that the actual mood status of the patients tested was to some extent related to the inflammatory gene expression. During a manic episode, the mRNA expression of 2/19 genes were significantly increased in monocytes of manic *vs.* euthymic BD patients; during depressive episodes 6/19 genes. In the presently extended series of 56 BD patients we largely confirmed this observation and now found 1/34 genes during mania ($n=7$ patients) and 6/34 genes during depression ($n=9$ patients) raised [in comparison to euthymic patients ($n=40$ patients), Table 5*b*]. Interestingly these were all cluster 2 genes. Although active disease thus is related to a higher expression of many of the cluster 2 signature genes (in depressive phases more than in manic phases), it must be noted that virtually all of the cluster 2 genes were still significantly higher in euthymic BD patients compared to HC.

Inflammatory gene expression at the protein level

We previously reported on the *IL1β*, *IL6*, *TNFα*, *CCL7* and *CCL2* levels in the serum of these sets of BD and SZ patients (Drexhage *et al*. 2008; Padmos *et al*. 2008*a*), and found that only *IL1β* was increased in BD (compared to HC), while *IL1β*, *IL6*, *TNFα* and *CCL2* were all increased in SZ patients (compared to HC).

With regard to *PTX3*, we were able in this study to measure serum levels and found these increased in BD and SZ patients (Fig. 2). The figure shows that in BD

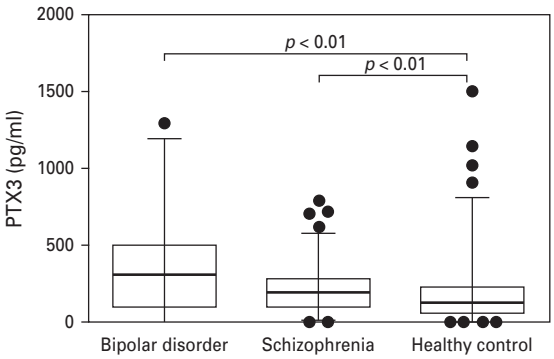

Fig. 2. Serum *PTX3* levels in bipolar disorder (BD) patients, schizophrenia (SZ) patients and healthy controls (HC). Box plots of *PTX3* are given. The serum *PTX3* level was determined via an in-house ELISA (M7M) on the serum of 58 BD patients, 181 SZ patients and 188 HC, some of whom were also used for Q-PCR according to the manufacturer's protocol (for patient details see Drexhage *et al*. 2008). The box indicates the lower and upper quartiles. The line within the box represents the median. The whiskers extend to the 2.5 and 97.5 percentiles. The outliers are characterized by the filled dots. ANCOVA was used for statistical evaluation. Age and gender were included in the statistical model, other confounding factors such as adiposity could, however, not be investigated, since we were not informed of the adiposity in the majority of the cases tested here. The figure shows that in BD and SZ, *PTX3* serum levels are significantly raised over HC levels, i.e. ~2.5-fold in BD and ~1.5-fold in SZ. Correlating serum *PTX3* protein levels to monocyte gene expression levels we found a significant positive correlation, although only weakly ($r = 0.184$, $p = 0.05$, Spearman's correlation).

and SZ *PTX3* serum levels are significantly raised over HC levels, i.e. ~2.5-fold in BD and ~1.5-fold in SZ. When we correlated serum *PTX3* protein levels to monocyte gene expression levels we found a significant positive correlation, although weakly ($r = 0.184$, $p = 0.05$, Spearman's correlation), indicating that factors other than monocyte gene expression also determine serum *PTX3* levels.

Discussion

The outcomes of our study show that circulating monocytes are set at a high inflammatory gene expression set-point in both SZ and BD. On the protein level these high gene expression set-points were (weakly, but significantly) reflected in high serum levels of pro-inflammatory cytokines and compounds.

Although monocytes of BD and SZ patients clearly overlapped in signature gene expression sets 1A and 1B, they also differed with regard to signature gene expression set 2. We found the MAP kinase-regulating factors *PTPN7* and *NAB2* up-regulated in monocytes of BD patients, but down-regulated in monocytes of SZ patients. Our immune biomarker approach thus made a distinction between BD and SZ possible. Although thus supporting the dichotomy between BD and SZ as introduced in 1899 by Kraepelin (1899), our immune data also lend support to the recently expressed view by geneticists (Bramon & Sham, 2001; Lichtenstein *et al*. 2009; Owen & Craddock, 2009; Thomson *et al*. 2005) that BD and SZ are strongly overlapping entities sharing the same vulnerability genes, since we found monocytes of considerable proportions of BD and SZ patients to share an up-regulated pro-inflammatory gene sub-cluster 1A, composed mainly of a network of well known pro-inflammatory cytokines and compounds such as *IL1*, *IL6*, *TNF*, *PTGS2/COX2* and *PTX3*, many of which have previously been found up-regulated mainly at the protein level in mood disorders and SZ (Drexhage *et al*. 2008; Padmos *et al*. 2008*a*).

In addition, we found monocytes, particularly of SZ patients, to be set at a further and higher inflammatory set-point, due in particular to an extra up-regulation of the transcription factors/regulators *EGR3*, *MXD1*, *MAFF* and *F3*. These transcription factors/regulators are mainly involved in proliferation and differentiation of monocytes, but also play a role in the regulation of the inflammatory set-point of monocytes/macrophages (Ayer & Eisenman, 1993; Blank, 2008; Carter & Tourtellotte, 2007; Collins *et al*. 2008; Hurlin & Huang, 2006; Motohashi *et al*. 1997). Our data, which show a strong correlation of *EGR3*, *MXD1*, *MAFF* and *F3* to the inflammatory cytokines and compounds to which *ATF3* and *DUSP2* were also correlating, supports such a view of involvement of these transcription factors in inflammation. Interestingly, the expression of *EGR3*, *MXD1*, *MAFF* and *F3* were particularly correlated to the up-regulation of the adipogenic and vascular pathology factors *THBS* and *SERPINB2/PAI-2*, and it is here of note that the incidence of the metabolic syndrome is increased in BD and SZ (Birkenaes *et al*. 2007).

Our study has limitations. First, patients with SZ were predominantly male and young in the 20-yr age group and virtually all had recent onset disease and were on antipsychotic medication; the BD patients were predominantly female and in the 40-yr age group, had mainly longstanding disease and many used lithium and/or antipsychotics. All patients were naturalistically treated and none of our patients was 'treatment naive'. It could be argued that age and gender simply explained the differences between SZ

and BD and that medication is the causal factor for the high inflammatory gene expression level in monocytes of psychiatric patients.

With regard to age and gender, it must be noted that we compared the patient findings with those of age- and gender-matched control groups and that the monocyte inflammatory state in these control groups of different age and gender did not differ (Supplementary Table 1).

With regard to the effects of medication it must be noted that effects of lithium and antipsychotics are generally anti-inflammatory in character (Drzyzga *et al*. 2006; Pollmacher *et al*. 2000; Rybakowski, 2000) and the data presented here, as well as our previous data (Padmos *et al*. 2008*a*), support such an immune suppressive action and show that, if anything, these medications do not induce but rather correct the abnormal inflammatory set-point of patient monocytes (see Table 5*a*). However, we can not totally rule out an important effect of illicit drugs on the induction of the specific characteristics of the SZ monocyte signature, since our study group of SZ patients was not controlled for this variable (the BD patients were, see Table 1).

Another limitation is that in our experimental design we made a selection of aberrantly expressed genes by selecting, in whole genome analysis, only highly over- and under-expressed genes (~3.5-fold), which were clearly involved in inflammation and inflammatory processes. Although this approach proved to be fruitful in detecting the three fingerprint patterns described here (which also made a distinction between BD and SZ possible), we may have missed important causal genes for the inflammatory set-points, since our assumption that the sheer expression level of genes is important for the inflammatory state, is naive. Clearly, further studies of additional genes that are less aberrantly expressed but are critically involved in inflammation and/or have previously been described as aberrant in psychiatric disorders are clearly needed to see whether they are essential components of the inflammatory monocyte gene fingerprints.

The criteria used in psychiatry for validating nosological categories have usually been restricted to clinical features, outcome and family history (Craddock & Owen, 2007). Kraepelin used these tools in formulating his ideas, leading to his dichotomous classification between SZ and BD. Given that the main goal of modern psychiatry is to provide effective treatment, the view has been expressed that the ultimate validator for a diagnostic system must be treatment response based on a detailed knowledge of pathogenesis (Owen & Craddock, 2009).

Our study, using powerful new research genomic tools, precisely provides such new immune biological validators, which probably not only play a role in the immune pathogenesis of SZ and BD but are also potential treatment targets. There are several reports indicating that pharmacological interferences with some of the up-regulated inflammatory signature genes found, i.e. interference with *PTGS2/COX-2*, *PDE4B* and *TNF*, may alleviate signs and symptoms of SZ and depression (Akhondzadeh *et al*. 2007; Martina *et al*. 2006; Muller *et al*. 2004, 2006; Myint *et al*. 2007; Nery *et al*. 2008; Tyring *et al*. 2006; Zhu *et al*. 2001) and it can thus be envisaged that in particular patients positive for monocyte cluster 1 genes would benefit from treatment with *COX-2*, *PDE4* and *TNF* inhibitors.

In conclusion, we here describe the first steps in an immune molecular dissecting approach on inflammatory monocytes, which has already led to the identification of three coherent sets of putative immune biomarker genes, opening new avenues for nosological distinctions in psychiatric disease based on inflammation. Our approach could also lead to putative sub-classification of patients with psychotic or BD, who could possibly benefit from adjunctive anti-inflammatory treatment targeting important fingerprint genes.

Note

Supplementary material accompanies this paper on the Journal's website (http://journals.cambridge.org/pnp).

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Statement of Interest

W. A. Nolen has received honoraria/speaker's fees from AstraZeneca, Eli Lilly, Pfizer, Servier and Wyeth, and has served on advisory boards for AstraZeneca, Cyberonics, Pfizer and Servier.

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